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AN OVIDUCAL ENZYME ISOLATED BY AFFINITY CHROMATOGRAPHY WHICH ACTS UPON THE VITELLINE ENVELOPE OF *BUFO ARENARUM* COELOMIC OOCYTES

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Summary

A trypsin-like oviducal proteinase acting upon the vitelline envelope of *Bufo arenarum* coelomic oocytes has been purified to apparent homogeneity by gel filtration on Sephadex G-200 and by affinity chromatography on a column of Sepharose 4-B containing covalently bound concanavalin A (Con A). The biologically active molecule migrated as a single band of protein upon SDS polyacrylamide gel electrophoresis.

During ovulation, amphibian oocytes are released from the ovary into the body cavity, thus receiving the name of body cavity or coelomic oocytes. Although many attempts have been made to fertilize such oocytes [1—3], little or no succes has been obtained unless the vitelline envelope has been previously altered, whether mechanically or by means of proteolytic enzymes [4, 5]. This would suggest that the vitelline coat would act as a barrier to fertilization [4]. Previous results obtained in our laboratories have shown that *Bufo arenarum* coelomic oocytes can be fertilized after they have passed through the portion of the oviduct known as pars recta (PR) [6]. Recently it has been definitively confirmed that coelomic oocytes can be fertilized successfully when they are treated with PR extract. In addition, we have partially isolated the active product of the crude extract, which resulted to be in a proteolytic-like enzyme acting upon the vitelline envelope [7]. Preliminary experiments designed to establish the mechanism of action of this protein during fertilization, indicate that the same can interact with concanavalin A (Con A), a metalloprotein isolated from the jack bean,

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Canavalia ensiformis [8]. These results suggest that affinity chromatography on immobilized Con A might be usable in the purification of this oviducal fertilization factor. In the present paper we report the purification of this molecule by affinity chromatography on Con A-Sepharose.

The procedure for the preparation of the PR extract has been described elsewhere [9]. The vitelline coat of *B. arenarum* coelomic oocytes is not attacked by sperm lysin. However, if the same oocytes are pretreated with a crude PR extract ("conditioned oocytes"), the vitelline envelope becomes sensible to sperm lysin. The bioassay used to estimate the activity of the active molecule during their purification is based on the above described principle. The bioassay was performed in plastic culture plates, with 0.5-ml wells at 25°C. 15 coelomic oocyte batches were conditioned in 0.5 ml of the fractions to be assayed over 30 min, being then transferred to 0.3 ml 10% Ringer containing 0.01 M Tris-HCl (pH 7.6) to which 2 μg/ml of sperm lysin prepared according to the method of Cabada [9] had been added. After 3 min the culture plates were observed under the stereoscopic microscope. Lysis degree of the vitelline envelope was compared with that of controls. Results were reported as 0 (no apparent egg alteration) to 4+ (enlargement and softening of the vitelline envelope accompanied with wrinkling of the egg surface and a decrease of egg tension). Results are expressed from 0 (no lysis) to 4+ (complete lysis). Con A (Sigma Chemical Co., St. Louis, Mo.) was conjugated to Sepharose 4-B (Pharmacia Fine Chemicals, Uppsala, Sweden) according to the procedure described by Cuatrecasas [10]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed as described by Laemmli [11] using Coomassie Blue stain. Protein content was determined by the procedure of Lowry et al. [12] with bovine serum albumin as a standard.

The active protein was isolated from the crude extract by two steps: gel filtration and affinity chromatography. The PR crude extract was directly applied to a column containing Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated and eluted with Ringer containing 0.01 M Tris-HCl (pH 7.4). Column dimensions were 50 × 2.5 cm and the void volume (V_0) was determined with Dextran Blue as a marker. Each 1.7-ml fraction was checked for adsorption at 280 nm and for biological activity. The active material from the Sephadex G-200 column was pooled and then applied to a column (0.7 × 6 cm) of Sepharose-bound Con A which had been equilibrated with Ringer containing 0.01 M Tris-HCl and 0.001 M each $MgCl_2$ and $MnCl_2$ (pH 7.4). The column was washed with this solution until 280 nm absorbance had fallen to a value below 0.05. The eluant was collected in 1-ml fractions at the rate of 1.5 ml/min. The bound material was then eluted with 0.2 M α-methyl-D-mannoside (Sigma Chemical Co., St. Louis, Mo.) (Fig. 1). In control column the specific sugar was added to the sample before chromatography (Fig. 2). The protein content of the pooled material of the void volume was nearly the same as that of the control column, indicating that only a very small fraction of the total protein had adsorbed. Identical results were obtained when the crude extract was directly chromatographed on the plant lectin column. SDS polyacrylamide gel electrophoresis of the PR crude extract was performed and the results are shown in Fig. 3. The Coomassie Blue stain revealed 11 distinct bands. On the other hand, when examined by SDS gel

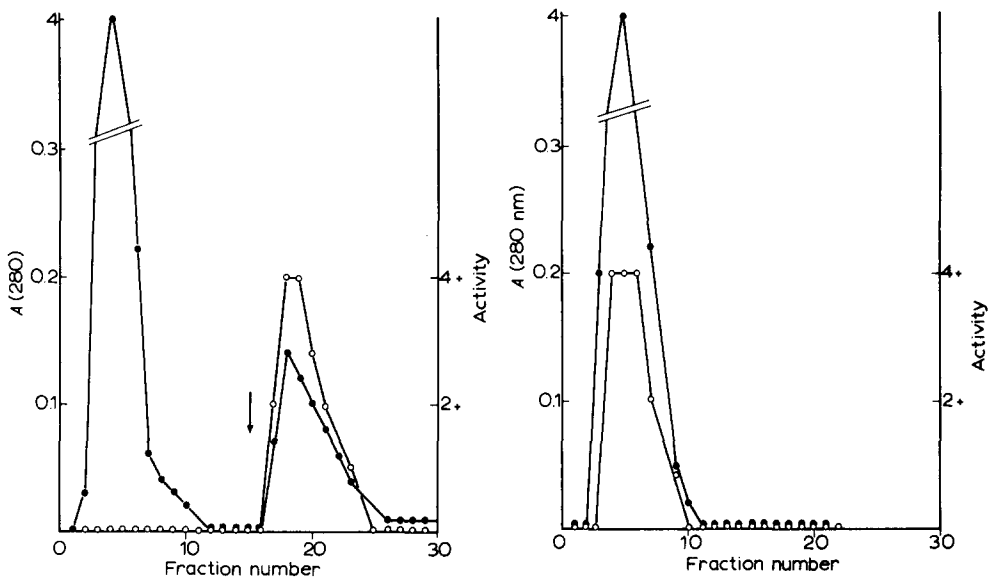

Fig. 1. Affinity chromatography of the active fraction eluted from Sephadex G-200 on a column of Con A bound to Sepharose 4-B. The column was washed with Ringer containing 0.01 M Tris-HCl and 0.001 M each $MgCl_2$ and $MnCl_2$ (pH 7.4) to remove material not bound to the Con A followed by elution with 0.2 M α-D-mannoside. The arrow denotes the point at which the specific sugar was added to the column. Fractions were assayed for activity (○—○) and for absorbance at 280 nm (●—●).

Fig. 2. Con A-Sepharose control column. 0.2 M α-methyl-D-mannoside was added to the sample before chromatography. Chromatographic conditions and symbols are as in Fig. 1.

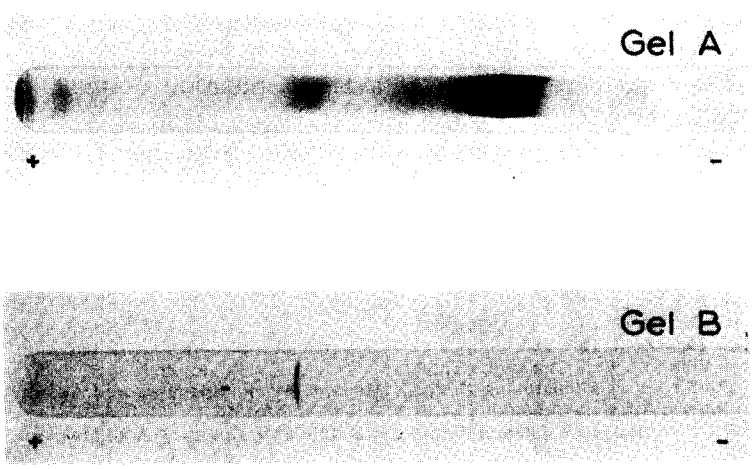

Fig. 3. SDS polyacrylamide gel electrophoresis of PR extract. Gel A, crude PR extract (200 μg of protein); Gel B, protein eluted from the Con A-Sepharose column (30 μg of protein). Electrophoresis was carried out on 7.5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. The gels were run in Tris-glycine buffer (pH 8.3) and stained with Coomassie Blue. Direction of the migration is from top (–) to bottom (+).

electrophoresis the material isolated from the Con A-Sepharose column exhibited only a single protein band (Fig. 3).

The data reported in this paper demonstrated that affinity chromatography using Con A covalently linked to agarose appears to be an useful tool

for the isolation of this proteolytic-like enzyme. This technique allows the recovery of high yields of the material and seems to possess a high degree of specificity. Inherent advantages of this method of purification are the rapidity and readiness of a potential single-step procedure. Con A affinity chromatography provides preliminar information on the molecular identity of this enzyme. Our data indicate that it is a glycoprotein since the active molecule binds to Con A-Sepharose and can be eluted with the appropriate specific sugar haptene. It has been shown that any glycoprotein possessing multiple α-D-mannopyranosyl or α-D-glucopyranosyl end groups or internal 2-*O*-linked α-D-mannopyranosyl residues will bind to Con A [13]. Lectins exhibit a high degree of discrimination between complex carbohydrates, can discriminate between stereoisomers and may give information about the type and position of substitution [14]. However, it should be noted that the reaction of lectins with oligosaccharides is highly complex depending not only on the type of monosaccharide present but also on their sequence and on the nature of the glycosidic linkages involved [15]. Thus, although the present study allows discrimination about this oviducal enzyme on the basis of its lectin interaction properties, further characterization of the carbohydrate content of this glycoprotein is necessary for the best knowledge of its function. Such experiments are presently in progress, and will provide additional information regarding the role of the female duct in the fertilization process.

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References

1 Rugh, R. (1935) J. Exp. Zool. 71, 163
2 Elinson, R.P. (1971) J. Exp. Zool. 176, 415
3 Wolf, D.P. and Hedrick, J.L. (1971) Dev. Biol. 25, 360
4 Elinson, R.P. (1973) J. Exp. Zool. 183, 291
5 Katagiri, Ch. (1974) J. Embryol. Exp. Morphol. 31, 573
6 Barbieri, F.D. (1971) In Recientes Adelantos en Biología (Mejía, R.H. and Moguilevsky, J.A., eds.), p. 402, Bona S.C.A., Buenos Aires
7 Miceli, D.C., Fernández, S.N., Raisman, J.S. and Barbieri, F.D. (1978) J. Embryol. Exp. Morphol., submitted for publication
8 Sumner, J.B. and Howell, S.F. (1936) J. Bacteriol. 32, 227
9 Cabada, M.O., Mariano, M.I. and Raisman, J.S. (1977) J. Exp. Zool., in the press
10 Cuatrecasas, P. (1970) J. Biol. Chem. 245, 3059
11 Laemmli, U.K. (1970) Nature 227, 680
12 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
13 Goldstein, I.J., Reichert, C.M., Misaki, A. and Gorin, A.J. (1973) Biochim. Biophys. Acta 317, 500
14 Sharon, N. and Lis, H. (1972) Science 177, 949
15 Kornfield, R. and Ferris, C. (1975) J. Biol. Chem. 250, 2614